Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, Nos 8–12, pp. 771–776, 1990
Printed in Great Britain

0731-7085/90 $3.00 + 0.00
Pergamon Press plc

Thermospray mass spectrometer as a quantitative specific, sensitive, detector for liquid chromatography. Its application to the analysis of pyridostigmine in human plasma*

S.L. MALCOLM,† M.J. MADIGAN and N.L. TAYLOR‡

Pharmacokinetics and Metabolism Department, Roche Products Ltd, Welwyn Garden City, Herts, UK

Abstract: A thermospray interface between a HPLC system and a mass spectrometer has been used to develop an assay for pyridostigmine bromide measuring plasma concentrations down to 1 ng ml^{-1} on a routine basis. Plasma is loaded onto an AASP reversed-phase cartridge, injected onto a HPLC system connected to a Finnigan 4500 mass spectrometer via a thermospray interface and the molecular ion monitored.

A deuterated internal standard is used, but calculations with and without the internal standard show its use does not materially improve the quantitation. The precision and accuracy at the limit of quantitation is $<\pm 5\%$ and 95–105%, respectively. The method is used to analyse samples from a bioavailability study by fully automated unattended overnight sample analysis.

Keywords: *Thermospray-mass spectrometry; HPLC analysis; pyridostigmine.*

Introduction

For some years now the thermospray interface between the ubiquitous liquid chromatographic system and the mass spectrometer has hovered on the brink of providing the sensitive, specific detector chromatographers have been searching for. It is widely used for compound identification, but as a quantitative technique its power is only slowly being realized. The high specificity, good sensitivity and wide linear response combine to make a powerful tool for quantitating HPLC analysis [1–4]. Whilst unwilling to describe it as a universally applicable detection system the authors would like to describe the sort of fully automated, quantitative work that can be achieved on such an instrument in a relatively short period of time.

Pyridostigmine bromide, is an inhibitor of acetylcholinesterase, which is used in anaesthesiology to reverse non-depolarizing neuromuscular blockade and also in the treatment of myasthenia gravis. Recent advances in the measurement of pyridostigmine have all employed solid-phase or cation exchange extraction and reversed-phase liquid chromatography with UV detection [5–9]. It is a quaternary ammonium compound with good HPLC characteristics but insufficient UV absorbance to enable the routine assay of plasma samples much below 5 ng ml^{-1}. A previous mass spectrometric method involved forming the *N*-demethylated product of pyridostigmine in the hot injector of a GLC and detection by chemical ionization and ion monitoring [10, 11]. The quantitation limit was 5 ng ml^{-1} from a 2-ml plasma sample.

A fully validated HPLC thermospray mass spectrometric method has been developed which can be used routinely down to 1 ng ml^{-1}. Human plasma (0.5 ml) is processed by a reversed-phase cartridge via an AASP, injected onto a reversed-phase HPLC system connected to a Finnigan 4500 mass spectrometer via a thermospray interface and the molecular ion monitored.

The method was used to analyse samples from a bioavailability study by fully automated unattended overnight sample analysis.

---

*Presented at the "Second International Symposium on Pharmaceutical and Biomedical Analysis", April 1990, York, UK.

†Author to whom correspondence should be addressed.
‡Present address: Glaxo Group Research, Greenford, Middlesex, UK.

Experimental

Standard solutions

Pyridostigmine bromide is hygroscopic and before weighing it was dried, under vacuum, over phosphorus pentoxide at 105°C, to a constant weight. Solutions of the drug and internal standard (d_6-pyridostigmine) were prepared in distilled water and stored at +4°C for periods of at least 1 month without deterioration.

Batch analyses

Calibration standards in the range 0.5–200 ng ml^{-1}, were prepared from pooled human plasma. QA samples were prepared by an independent analyst, to monitor assay performance. All standards, QAs and samples not analysed immediately were stored at −80°C.

Sample work up

AASP C_8 cartridges were primed with 1 ml methanol and 0.01 M sodium chloride. The plasma samples were centrifuged at 3000 rpm for 10 min and an aliquot (0.5 ml) loaded onto a cartridge with internal standard (=10 ng ml^{-1}) and 0.01 M sodium chloride (0.5 ml) under nitrogen pressure. The cartridges were washed with 0.01 M sodium chloride (0.5 ml) without allowing them to dry out. The cassettes were transferred to the AASP and individually purged with 5 × 25 μl of 50% methanol–0.01 M sodium chloride before injection (1 min valve reset time).

HPLC system

The analytical column was a Waters C_{18} NovaPak cartridge in a RCM module, protected by a Guard-Pak containing a C_{18} μBondaPak cartridge. The eluent was methanol–0.1 M ammonium acetate (11:89, v/v); containing 0.0625 mM tetramethyl ammonium chloride and 0.2% triethylamine, pH adjusted to 6.6 with glacial acetic acid. It was degassed continuously with helium through a 6-μm solvent filter and the flow through the column was 1.5 ml min^{-1}. The retention time of pyridostigmine was 2.6 min but the total run time was 21 min, to exclude interferences from late running peaks.

Thermospray/mass spectrometer

A Finnigan MAT 4500 mass spectrometer with a thermospray interface was used as the detection system. The INCOS data system controlled the acquisition of data from the mass spectrometer, provided the signal for the injections to the AASP and was used to integrate the raw data. The thermospray vaporizer was 145°C, the jet (source) temperature was 200°C and the ions monitored were *m/z* 181 pyridostigmine-d_0 (0.8 s), and *m/z* 187 pyridostigmine-d_6 (0.2 s). The mass spectrometer was focused using the ion *m/z* 116 (identity unknown) present in the background spectrum.

Data handling

Peak height data were transferred to an in-house suite of programs on an IBM PS/2 computer for curve fitting and calculation of concentrations of unknowns. Standard curves were generated by weighted (using the calculated response) linear regression analysis of the peak height ratio of drug to internal standard or of drug alone, the coefficients of the equation used to determine the back calculated values of the standards, the QA samples and the unknowns.

Stability

A large volume of blank plasma was spiked at two concentrations and divided into 0.6-ml fractions. Five replicates were analysed immediately and after storage at −80°C for 1 month.

Specificity

Several pooled human plasma samples and individual pre-dose samples were monitored for interfering peaks.

Recovery

The recovery through the sample work up as well as any effect of blood collection device on drug partitioning was monitored.

Clinical samples

A relative bioavailability crossover study of a 120 mg solution of pyridostigmine versus 2 × 60 mg tablets was carried out in six volunteers. Blood samples were collected at various times after dosing up to 24 h and the plasma stored frozen until analysed.

Results

The data from several standard curves (n = 4) containing QA samples is presented in Table 1 where the back calculated values of the

Table 1
Assay performance data for standards and QA samples calculated with and without the internal standard

Sample concentration ($ng\ ml^{-1}$)	n		Mean		% Error		Precision (RSD)	
	With	Without	With	Without	With	Without	With	Without
0.5	2	2	0.52	0.51	4.1	2.4	2.5	0.4
1	4	4	0.99	1.01	−1.0	1.0	4.8	6.8
2	4	4	1.97	1.92	−1.5	−4.2	6.6	9.1
5	4	4	4.51	4.52	−9.8	−9.6	8.8	10.1
10	4	4	9.77	9.84	−2.3	−1.6	2.2	11.1
20	4	3	19.2	17.1	−4.1	−14.4	4.5	10.4
50	4	3	51.3	49.1	2.6	−1.9	6.6	7.3
100	4	4	106.7	113.1	6.7	13.1	3.7	0.9
200	4	4	214.7	223.0	7.3	11.5	2.7	17.3
						mean	4.7	8.2
QA1 8.90	8	8	8.55	8.16	−3.9	−8.3	10.5	6.9
QA2 77.0	8	8	78.1	85.8	1.3	11.4	7.4	17.0
						mean	9.0	12.0

standards and the results of the QA samples have been used to determine the accuracy and precision of the method. Although in these experiments the results at 0.5 ng ml^{-1} were perfectly acceptable, after routine analysis of large batch sizes the lowest standard often fell outside limits of acceptability (the back calculated value ±15% of the theoretical) therefore the quantitation limit was defined as 1 ng ml^{-1}.

These results also show that the method is acceptable although not as precise when the data were calculated without the internal standard. The precision and accuracy at this quantitation limit with the internal standard was +4.8 and 99% and without it was 6.8 and 99%, respectively (n = 4). The overall precision of the method was +4.7 with and 8.2% without the internal standard and the accuracy of all the standards fell within 95–105% of the true values. Using the QA samples the accuracy of the assay ranged from 91.7 to +111.4% (n = 8). The results from the stability study are shown in Table 2 and show there were no detectable losses after storage at −80°C for 1 month.

Figure 1 shows typical traces of the ions monitored for a pre-dose sample and for a 0.5 ng ml^{-1} standard. All the blank plasma samples monitored looked much the same with no discernible peak at the retention time of pyridostigmine. Figure 2 shows the mass spectrum of the internal standard around the molecular ion. The levels of d_0 material (m/z 181) from the internal standard were 0.01% of the d_6 (m/z 187) peak and therefore did not cause any interference at the concentration used (=10 ng ml^{-1}).

Table 2
Stability of pyridostigmine in plasma after storage at −80°C

	Initial concentration ($ng\ ml^{-1}$)	Analysis after 1 month
	55.6	55.8
	52.9	56.3
	56.5	54.1
	51.4	59.0
	54.8	54.5
mean	54.2	55.9
	3.48	3.80
	3.29	3.65
	3.64	3.56
	3.81	3.58
	3.84	3.44
mean	3.61	3.61

The recovery of pyridostigmine throughout the sample work up was determined over the entire concentration range (0.5–200 ng ml^{-1}) and the mean value was 87%.

There was no difference between the results from spiking blood in heparinized Vacutainers and clean glass containers with Teflon lids, therefore it was assumed the heparin and rubber stopper had no effect on recovery.

The method was applied to the analysis of pyridostigmine in plasma from a relative bioavailability study in man. The mean data for each treatment is shown in Fig. 3. Although concentrations were higher at the first time point after the solution formulation, the tablets gave higher plasma concentrations.

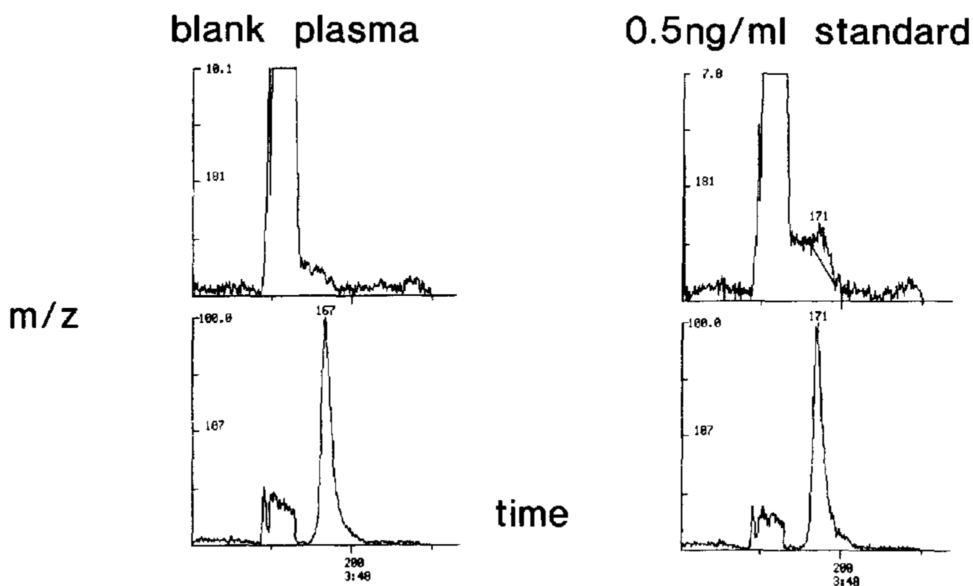

Figure 1
Typical ion traces of a blank plasma sample (volunteers pre-dose plasma) containing internal standard and a 0.5 ng ml^{-1} plasma standard, *m/z* 181 for pyridostigmine and *m/z* 187 for the d_6 material.

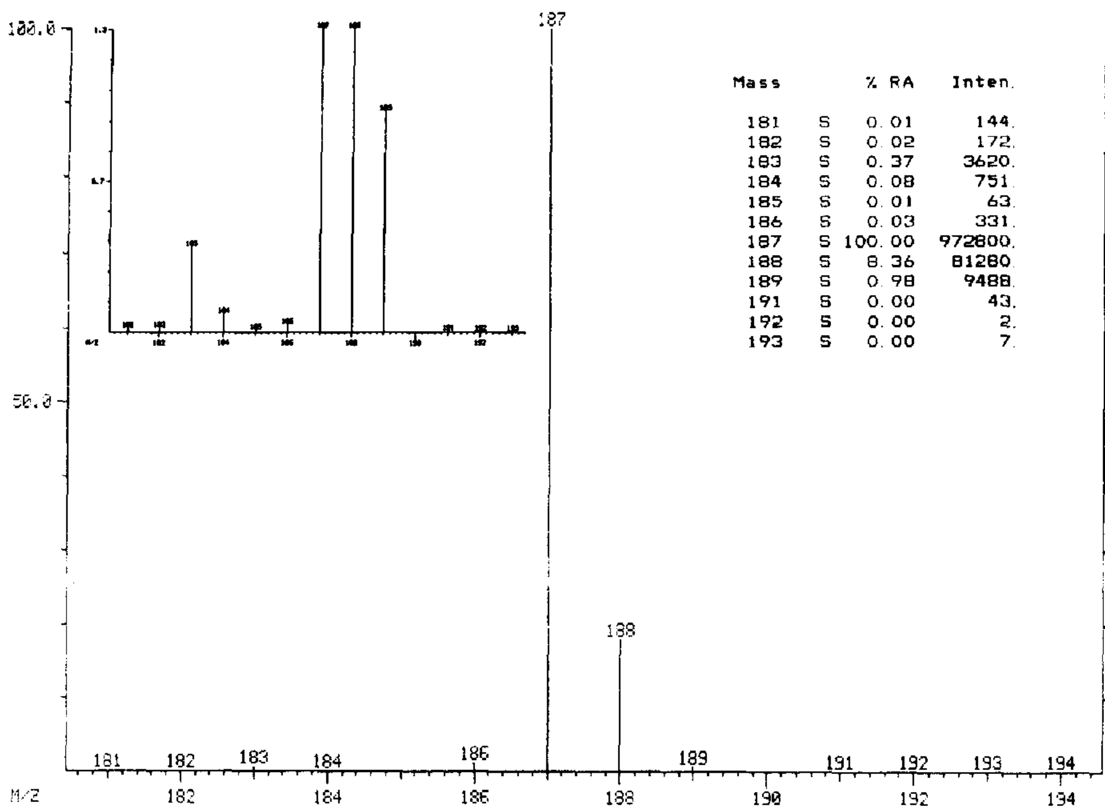

Figure 2
Thermospray mass spectrum of the d_6 pyridostigmine used as internal standard, showing only the masses around the parent ion. Inset is the same spectrum magnified to show the levels of *m/z* 181 in the spectrum, and each ion's percent of *m/z* 187 (% RA = relative abundance).

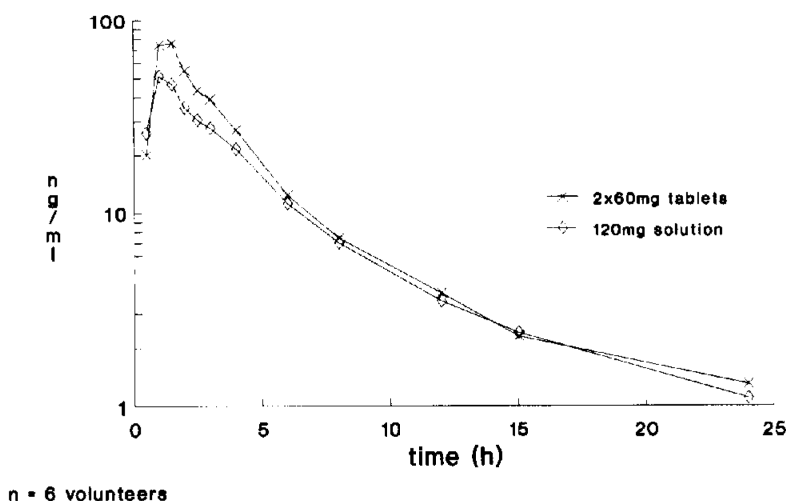

Figure 3
Mean plasma concentrations of pyridostigmine (ng ml^{-1}) from six volunteers each receiving 120 mg pyridostigmine in solution crossed over with 2 × 60 mg pyridostigmine tablets.

Discussion

Although the mass ion of the analyte is low (*m/z* 181), causing increased risk of background ions and therefore noise, it has been possible to develop an analytical method with quite impressive sensitivity. It must be admitted, however, that the noise at this ion is normally quite significant. It was the use of tetramethyl ammonium chloride in the eluent which was successful in suppressing the background, thereby improving sensitivity. The high background is probably due to solvent and adduct ions of low molecular weight, and it is assumed that the tetramethyl ammonium chloride has a competitive or disruptive effect on these processes. The final concentration in the eluent was a compromise between suppression and condensation, because this, like other ion-pair reagents, is non-volatile and over time causes blockages in the source. No problems were encountered at the concentration used in the present assay.

The triethylamine in the eluent blocked the active free silanol sites of the column and ensured a good sharp peak for quantitation. Since it is a volatile solvent it does not cause any condensation in the ion source and had no effect on the thermospray response of pyridostigmine. However, its addition elevates the pH of the eluent to a point where dissolution of silica becomes a problem. It was decided to reduce the pH rather than use a silica sparge column because the saturated eluent leaves a silica deposit in the source causing blockages and reducing sensitivity with time.

Although the quaternary ammonium compound is very polar its properties on a reversed-phase system were unusual. The compound is highly retained in simple solvent–water mixtures, hence the ability to purge with 50% methanol with no losses. It is the presence of the ammonium acetate in the eluent which causes it to elute at this retention time. Presumably the ammonium ion competes with the compound for absorption. It was initially felt that this was a normal phase process but the addition of triethylamine to block these sites had no effect on retention time, but significantly improved the peak shape.

The high organic content of the purge eluent was found to be necessary in order to "wet" the cartridges before injection. After transferring the cassettes to the AASP it was some time before the final injections were made (16–18 h) and the cartridges completely dried out. This wetting allowed satisfactory recovery from all of the cartridges.

Although it was fortunate that an ideal internal standard was available for this method, namely the deuterated material, the stability of the thermospray ionization was found to be sufficient to enable the response from the drug alone without any reference material. This is quite standard procedure for solid-phase methods with more traditional detection, but it has been considered essential for mass spectrometric methods and particu-

larly for thermospray that internal standards are included. Other than the fact that it is necessary to use a complicated eluent system that is tailored to this form of ionization no immediate explanation for this stability is apparent.

Conclusions

The described method can quantitate pyridostigmine down to 0.5–1 ng ml^{-1} using 0.5 ml plasma.

It is a simple method although it does use sophisticated instrumentation. In this case the stability of the thermospray ionization does not warrant an internal standard although the results are improved by its use.

It is demonstrated that low mass ions, with increased risk of background, can be used for sensitive quantitation.

The instrumentation allows for overnight unattended operation allowing reasonably large batch sizes.

References

[1] C. Lindberg and J. Paulson, 5th Montreux meeting on LC–MS, SFC–MS, MS–MS, Freiburg (1988).
[2] H.J. Pieniaszek, H.-S.L. Shen, D.M. Garner, G.O. Page, L.M. Shalaby, R.K. Isensee and C.C. Whitney, *J. Chromatogr.* **493**, 79–92 (1989).
[3] R.D. Voyksner and T.W. Pack, *Biomed. Environ. Mass Spectrom.* **18**, 897–903 (1989).
[4] W.J. Blanchflower and D.G. Kennedy, *Biomed. Environ. Mass Spectrom.* **18**, 935–936 (1989).
[5] M.G.M. De Ruyter, R. Cronelly and N. Castagnoli, *J. Chromatogr.* **183**, 193–201 (1980).
[6] R.I. Ellin, P. Zvirblis and M.R. Wilson, *J. Chromatogr.* **228**, 235–244 (1982).
[7] M.L. Shih and R.I. Ellin, *Methods of Sorbent Extraction*, Analytichem International, M251.
[8] O. Yturralde, R.-Y. Lee, L.Z. Benet, L. Fleckerstein and E.T.L. Lin, *J. Liquid Chromatogr.* **10**, 2231–2246 (1987).
[9] H. Matsunaga, T. Suehiro, T. Saita, Y. Nakano, M. Mori, K. Takara and K. Oda, *J. Chromatogr.* **422**, 353–355 (1987).
[10] K. Chan, N.E. Williams, J.D. Baty and T.N. Calvey, *J. Chromatogr.* **120**, 349–358 (1976).
[11] S.-M. Aquilonius, S.-A. Eckernas, P. Hartvig, B. Lindstrom and P. Osterman, *Eur. J. Clin. Pharmacol.* **18**, 423–428 (1980).

[*Received for review 5 April 1990*]